PROCESS FOR THE HETEROTROPHIC PRODUCTION OF MICROBIAL PRODUCTS WITH HIGH CONCENTRATIONS OF OMEGA-3 HIGHLY UNSATURATED FATTY ACIDS

COMPOSITION OF MATTER AND PROCESS

Cross-Reference to Related Applications

This application is a continuation-in-part of copending and commonly assigned U.S. patent application Serial No. 07/439,093, filed November 17, 1989, and entitled "Process for Heterotrophic Production of Microbial Products with High Concentrations of Omega-3 Highly Unsaturated Fatty Acids" now abandoned which is incorporated herein in its entirety by reference and is a continuation-in-part of U.S. Patent Application Serial No. 07/241,410, filed September 7, 1988, and entitled "Process for Heterotrophic Production of Microbial Products with High Concentrations of Omega-3 Highly Unsaturated Fatty Acids" which was previously expressly abandoned.

Field of the Invention

The field of this invention relates to heterotrophic organisms and a process for culturing them for the production of lipids with high concentrations of omega-3 highly unsaturated fatty acids (HUFA) suitable for human and animal consumption as food additives or for use in pharmaceutical and industrial products.

Background of the Invention

Omega-3 highly unsaturated fatty acids are of significant commercial interest in that they have been recently recognized as important dietary compounds for preventing arteriosclerosis and coronary heart disease, for alleviating inflammatory conditions and for retarding the growth of tumor cells. These beneficial effects are a result both of omega-3 highly unsaturated fatty acids causing competitive inhibition of compounds produced from omega-6 fatty acids, and from beneficial compounds produced directly from the omega-3 highly unsaturated fatty acids themselves (Simopoulos et al.,

1986). Omega-6 fatty acids are the predominant highly unsaturated fatty acids found in plants and animals. Currently the only commercially available dietary source of omega-3 highly unsaturated fatty acids is from certain fish oils which can contain up to 20-30% of these fatty acids. The beneficial effects of these fatty acids can be obtained by eating fish several times a week or by daily intake of concentrated fish oil. Consequently large quantities of fish oil are processed and encapsulated each year for sale as a dietary supplement.

However, there are several significant problems with these fish oil supplements. First, they can contain high levels of fat-soluble vitamins that are found naturally in fish oils. When ingested, these vitamins are stored and metabolized in fat in the human body rather than excreted in urine. High doses of these vitamins can be unsafe, leading to kidney problems or blindness and several U.S. medical associations have cautioned against using capsule supplements rather than real fish. Secondly, fish oils contain up to 80% of saturated and omega-6 fatty acids, both of which can have deleterious health effects. Additionally, fish oils have a strong fishy taste and odor, and as such cannot be added to processed foods as a food additive, without negatively affecting the taste of the food product. Moreover, the isolation of pure omega-3 highly unsaturated fatty acids from this mixture is an involved and expensive process resulting in very high prices ($200-$1000/g) for pure forms of these fatty acids (Sigma Chemical Co., 1988; CalBiochem Co., 1987).

The natural source of omega-3 highly unsaturated fatty acids in fish oil is algae. These highly unsaturated fatty acids are important components of photosynthetic membranes. Omega-3 highly unsaturated fatty acids accumulate in the food chain and are eventually incorporated in fish oils. Bacteria and yeast are not able to synthesize omega-3 highly

unsaturated fatty acids and only a few fungi are known which can produce minor and trace amounts of omega-3 highly unsaturated fatty acids (Weete, 1980; Wassef, 1977; Erwin, 1973).

Algae have been grown in outdoor cultivation ponds for the photoautotrophic production of a wide variety of products including omega-3 highly unsaturated fatty acid containing biomass. For example, U.S. patent 4,341,038 describes a method for the photosynthetic production of oils from algae, and U.S. patent 4,615,839 describes a process for concentrating eicosapentaenoic acid (EPA) (one of the omega-3 highly unsaturated fatty acids) produced photosynthetically by strains of the green alga Chlorella. Photoautotrophy is the process whereby cells utilize the process of photosynthesis to construct organic compounds from CO_2 and water, while using light as an energy source. Since sunlight is the driving force for this type of production system, algal cultivation ponds require large amounts of surface area (land) to be economically viable. Due to their large size, these systems cannot be economically covered, because of high costs and technical problems, and because even transparent covers tend to block a significant amount of the sunlight. Therefore, these production systems are not axenic, and are difficult to maintain as monocultures. This is especially critical if the cultures need to be manipulated or stressed (e.g. nitrogen limited) to induce production of the desired product. Typically, it is during these periods of stress, when the cells are only producing product and are not multiplying, that contaminants can readily invade the cultures. Thus, in most cases, the biomass produced is not desirable as a food additive for human consumption without employing expensive extraction procedures to recover the lipids. Additionally, photosynthetic production of algae in outdoor systems is very costly, since cultures must be maintained at low densities (1-2g/l) to prevent light limitation of the

culture. Consequently, large volumes of water must be processed to recover small quantities of algae, and since the algal cells are very tiny, expensive harvesting processes must also be employed.

Mixotrophy is an alternative mode of production whereby certain strains of algae carry on photosynthesis with light as a necessary energy source but additionally use organic compounds supplied in the medium. Higher densities can be achieved by mixotrophic production and the cultures can be maintained in closed reactors for axenic production. U.S. patents 3,444,647 and 3,316,674 describe processes for the mixotrophic production of algae. However, because of the need to supply light to the culture, production reactors of this type are very expensive to build and operate, and culture densities are still very limited.

An additional problem with the cultivation of algae for omega-3 highly unsaturated fatty acid production, is that even though omega-3 highly unsaturated fatty acids comprise 20-40% of some strains' total fatty acids, the total fatty acid content of these algae is generally very low, ranging from 5-10% of ash-free dry weight. In order to increase the fatty acid content of the cells, they must undergo a period of nitrogen limitation which stimulates the production of lipids. However, of all the strains noted to date in the literature, and over 60 strains evaluated by the inventor, all exhibit a marked decrease in omega-3 highly unsaturated fatty acids as a percentage of total fatty acids, when undergoing nitrogen limitation (Erwin, 1973; Pohl & Zurheide, 1979).

With respect to economics and to utilizing omega-3 highly unsaturated fatty acids as a food additive, it would be desirable to produce these fatty acids in a heterotrophic culture. Heterotrophy is the capacity for sustained and continuous growth and cell division in the dark in which both energy and cell carbon are obtained solely from the metabolism of an organic substrate(s).

Since light does not need to be supplied to a heterotrophic culture, the cultures can be grown at very high densities in closed reactors. Heterotrophic organisms are those which obtain energy and cell carbon from organic substrates, and are able to grow in the dark. Heterotrophic conditions are those conditions that permit the growth of heterotrophic organisms, whether light is present or not. However, the vast majority of algae are predominantly photoautotrophic, and only a few types of heterotrophic algae are known. U.S. patents 3,142,135 and 3,882,635 describe processes for the heterotrophic production of protein and pigments from algae such as Chlorella, Spongiococcum, and Prototheca. However these genera and others that have been documented to grow very well heterotrophically (e.g. Scenedesmus), do not produce omega-3 highly unsaturated fatty acids (Erwin, 1973). The very few heterotrophic algae known to produce any omega-3 highly unsaturated fatty acids (e.g., apochlorotic diatoms or apochlorotic dinoflagellates) generally grow slowly and produce low amounts of omega-3 highly unsaturated fatty acids as a percentage of ash-free dry weight (Harrington and Holtz, 1968; Tornabene et al., 1974).

A few higher fungi are known to produce omega-3 highly unsaturated fatty acids, but they comprise only a very small fraction of the total fatty acids in the cells (Erwin, 1973; Wassef, 1977; Weete, 1980). As such, they would not be good candidates for commercial production of omega-3 highly unsaturated fatty acids. For example, Yamada et al. (1987) recently reported on the cultivation of several species of the fungus, Mortierella, (isolated from soils) for the production of the omega-6 fatty acid, arachidonic acid. These fungi also produce small amounts of omega-3 eicosapentaenoic acid along with the arachidonic acid when grown at low temperatures (5-24°C). However, the resulting eicosapentaenoic acid content was only 2.6% of the dry weight of the cells, and the low temperatures necessary

to stimulate production of this fatty acid in these species would result in greatly decreased productivities (and economic potential) of the cultivation system. Some single-celled members of the order Thraustochytriales are also known to produce omega-3 highly unsaturated fatty acids (Ellenbogen, 1969, Wassef, 1977; Weete, 1980; Findlay et al., 1986) but they are known to be difficult to culture. Sparrow (1960) noted that the minuteness and simple nature of the thalli of the family Thraustochytriaceae (order Thraustochytriales) make them exceedingly difficult to propagate. Additional reasons for this difficulty have been outlined by Emerson (1950) and summarized by Schneider (1976): "1) these fungi consist of very small thalli of only one or a few cells, which generally grow very slowly in culture, and are very sensitive to environmental perturbation; 2) they are generally saprophytes, or parasites with very specialized nutritional and environmental demands; and 3) in pure culture they generally exhibit restricted growth, with vegetative growth terminating after a few generations." (Although some prior art classifies the thraustochytrids as fungi, the most recent consensus is that they should be classified as algae, see discussion below.)

As a result little attention has been paid to the numerous orders of these microorganisms, and those studies that have been conducted, have been predominantly carried out with a taxonomic or ecological focus. For example, even though the simple fatty acid distribution of several members of the order Thraustochytriales has been reported from a taxonomic perspective (Ellenbogen, 1969); Findlay et al., 1986), no one has ever reported their total fatty acid content or lipid content as percent dry weight. Unless data on the total lipid content is available, one cannot evaluate an organism's potential for use in the production of any type of fatty acid. For example, the omega-3 highly unsaturated fatty acid content of the

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lipids of some marine macroalgae (seaweeds) is reported to be very high, up to 51% of total fatty acids (Pohl & Zurheide, 1979). However, the lipid content of macroalgae is typically very low, only 1-2% of cellular dry weight (Ryther, 1983). Therefore, despite the reported high content of omega-3 highly unsaturated fatty acids in the fatty acids of macroalgae, they would be considered to be very poor candidate organisms for the production of omega-3 highly unsaturated fatty acids. Despite a diligent search by the inventor, no reports of simple proximate analysis (% protein, carbohydrate and lipid) of the Thraustochytriales has been found, nor has anyone reported attempts to cultivate these species for purposes other than laboratory studies of their taxonomy, physiology or ecology. Additionally, many of the strains of these microorganisms have been isolated by simple pollen baiting techniques (e.g., Gaertner, 1968). Pollen baiting techniques are very specific for members of the Thraustochytriales, but do not select for any characteristics which may be desirable for large scale cultivation of microorganisms.

Thus, until the present invention, there have been no known heterotrophic organisms suitable for culture that produce practical levels of omega-3 highly unsaturated fatty acids and such organisms have been thought to be very rare in the natural environment.

Brief Summary of the Invention

The present invention is directed toward a food product with a high concentration of omega-3 highly unsaturated fatty acids (HUFAs) which includes microorganisms characterized by having a high concentration of fatty acids of which a high percentage are omega-3 highly unsaturated fatty acids. In addition or alternatively, the food product can include omega-3 HUFAs extracted from the microorganisms. Specifically, the microorganisms are Thraustochytriales, namely,

Thraustochytrium or Schizochytrium. The microorganisms or extracted omega-3 HUFAs are incorporated with additional food material which may be either animal food or human food. The food product of the present invention may have the bioavailability of the omega-3 HUFAs contained therein increased by lysing the cells of the microorganisms. The food product may also be extruded. In order to prevent degradation of the omega-3 HUFAs, the food product may be packaged under non-oxidizing conditions or may further comprise an antioxidant.

Another embodiment of the present invention relates to a method of raising an animal comprising feeding the animal Thraustochytriales or omega-3 HUFAs extracted therefrom. Animals raised by the method of the present invention include poultry, cattle, swine and seafood, which includes fish, shrimp and shellfish. The omega-3 HUFAs are incorporated into the flesh, eggs and other products of these animals which are consumed by humans.

Omega-3 HUFAs may be consumed as the whole cell microbial product, the extracted omega-3 HUFA product, or the animal or animal product incorporating omega-3 HUFAs. Increased intake of omega-3 HUFAs produced in accordance with the present invention by humans is effective in preventing or treating cardiovascular diseases, inflammatory and/or immunological diseases, and cancer.

Yet another embodiment of the present invention is a method of producing omega-3 HUFAs which comprises culturing Thraustochytriales in a medium with a source of organic carbon and assimilable nitrogen. Preferably, the source of organic carbon and assimilable nitrogen comprises ground grain. The method further comprises culturing Thraustochytriales consisting of Thraustochytrium, Schizochytrium, or mixtures thereof under nutrient-limited or nitrogen-limited conditions for an effective amount of time, preferably about 6 to about 24 hours, and harvesting the Thraustochytriales

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during the period of nitrogen limitation in order to increase the concentration of omega-3 HUFAs in the microorganisms. The method further comprises adding an antioxidant compound selected from the group consisting of BHT, BHA, TBHQ, ethoxyquin, beta-carotene, vitamin E and vitamin C during post-harvest processing in order to prevent degradation of the omega-3 HUFAs. The method further comprises stressing the microorganisms with low temperatures during culturing, maintaining a high dissolved oxygen concentration in the medium during culturing, and adding to the medium effective amounts of phosphorous and a microbial growth factor (yeast extract or corn steep liquor) to provide sustained growth of the microorganisms. The present method further includes culturing unicellular microorganisms having the identifying characteristics of ATCC Nos. 20888, 20889, 20890, 20891, 20892 and mutant strains derived therefrom. Omega-3 HUFAs produced by the method can then be separated from the lipids extracted from the microorganisms by fractional crystallization which comprises rupturing the microorganism cells, extracting the lipid mixture from the ruptured cells with a solvent, hydrolyzing the lipid mixture, removing non-saponifiable compounds and cold-crystallizing the non-HUFAs in the lipid mixture.

A further embodiment of the present invention is a method for selecting unicellular, aquatic microorganisms capable of heterotrophic growth and capable of producing omega-3 HUFAs comprising selecting microorganisms of a size between about 1μm and 25μm from a small population of microorganisms collected from naturally occuring shallow saline habitats, culturing the microorganisms in a medium comprising organic carbon, assimilable nitrogen, assimilable phosphorous and a microbial growth factor under heterotrophic conditions, and selecting clear, white, orange, or red-colored non-filamentous colonies having rough or textured surfaces.

Detailed Description of the Preferred Embodiments

For purposes of definition throughout the application, it is understood herein that a fatty acid is an aliphatic monocarboxylic acid. Lipids are understood to be fats or oils including the glyceride esters of fatty acids along with associated phosphatides, sterols, alcohols, hydrocarbons, ketones, and related compounds.

A commonly employed shorthand system is used in this specification to denote the structure of the fatty acids (e.g., Weete, 1980). This system uses the letter "C" accompanied by a number denoting the number of carbons in the hydrocarbon chain, followed by a colon and a number indicating the number of double bonds, i.e., C20:5, eicosapentaenoic acid. Fatty acids are numbered starting at the carboxy carbon. Position of the double bonds is indicated by adding the Greek letter delta (Δ) followed by the carbon number of the double bond; i.e., C20:5omega-3$\Delta^{5,8,11,14,17}$. The "omega" notation is a shorthand system for unsaturated fatty acids whereby numbering from the carboxy-terminal carbon is used. For convenience, w3 will be used to symbolize "omega-3," especially when using the numerical shorthand nomenclature described herein. Omega-3 highly unsaturated fatty acids are understood to be polyethylenic fatty acids in which the ultimate ethylenic bond is 3 carbons from and including the terminal methyl group of the fatty acid. Thus, the complete nomenclature for eicosapentaenoic acid, an omega-3 highly unsaturated fatty acid, would be C20:5w3$\Delta^{5,8,11,14,17}$. For the sake of brevity, the double bond locations ($\Delta^{5,8,11,14,17}$) will be omitted. Eicosapentaenoic acid is then designated C20:5w3, Docosapentaenoic acid (C22:5w3$\Delta^{7,10,13,16,19}$) is C22:5w3, and Docosahexaenoic acid (C22:6w3$\Delta^{4,7,10,13,16,19}$) is C22:6w3. The nomenclature "highly unsaturated fatty acid" means a fatty acid with

4 or more double bonds. "Saturated fatty acid" means a fatty acid with 1 to 3 double bonds.

A collection and screening process was developed by the inventor to readily isolate many strains of microorganisms with the following combination of economically desirable characteristics for the production of omega-3 highly unsaturated fatty acids: 1) capable of heterotrophic growth; 2) high content of omega-3 highly unsaturated fatty acids; 3) unicellular; 4) preferably low content of saturated and omega-6 highly unsaturated fatty acids; 5) preferably nonpigmented, white or essentially colorless cells; 6) preferably thermotolerant (ability to grow at temperatures above 30°C); and 7) preferably euryhaline (able to grow over a wide range of salinities, but especially at low salinities).

Collection, isolation and selection of large numbers of suitable heterotrophic strains can be accomplished by the following method. Suitable water samples and organisms typically can be collected from shallow, saline habitats which preferably undergo a wide range of temperature and salinity variation. These habitats include marine tide pools, estuaries and inland saline ponds, springs, playas and lakes. Specific examples of these collection sites are: 1) saline warm springs such as those located along the Colorado river in Glenwood Springs, Colorado, or along the western edge of the Stansbury Mountains, Utah; 2) playas such as Goshen playa located near Goshen, Utah; 3) marine tide pools such as those located in the Bird Rocks area of La Jolla, California; and 4) estuaries, such as Tiajuana estuary, San Diego County, California. Special effort should be made to include some of the living plant matter and naturally occurring detritus (decaying plant and animal matter) along with the water sample. The sample can then be refrigerated until return to the laboratory. Sampling error is minimized if the water sample is shaken for 15-30 seconds, prior to pipetting

or pouring a portion, for example, 1-10 ml, into a filter unit. The filter unit includes 2 types of filters: 1) on top, a sterile Whatman #4 filter (Trademark, Whatman Inc., Clifton, N.J.); and 2) underneath the Whatman filter, a polycarbonate filter with 1.0μm pore size. The purpose of the first (top) filter is to remove all particulate matter greater than about 25μm, generally allowing only unicellular type material to pass onto the 1.0μm polycarbonate filter. The first filter greatly reduces the number of mold colonies that subsequently develop upon incubation of the polycarbonate filter at elevated temperatures, thereby enhancing the opportunities for other colonies to develop. Mold spores are very numerous in coastal and inland saline waters, and mold colonies can quickly cover an agar plate unless screened out. The 1.0μm size of the polycarbonate filter is chosen to allow many of the bacteria to pass on through into the filtrate. The purpose of using a sandwich filter design is to select for unicellular organisms at least a portion of whose cells range in diameter from about 1μm to about 25μm in size (organisms which could potentially be grown easily in a fermenter system for production on a large scale). Extensive growth of these unicellular organisms can be encouraged by incubation of the polycarbonate filter on an agar plate. Competition between organisms growing on the filter facilitates the isolation of competitive, robust strains of single-celled microorganisms. Unicellular aquatic microorganisms selected by the foregoing method display a range of cell size depending on growth conditions and stage of reproductive cycle. Most cells in culture have diameters in the range from about 1μm to about 25μm; however, cells (thalli and sporangia) in the cultures can be found that have larger diameters (depending on the strain) up to about 60μm.

After filtration, the polycarbonate filter can be placed on an agar plate containing saline media containing a source of organic carbon such as

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carbohydrate including glucose, various starches, molasses, ground corn and the like, a source of assimilable organic or inorganic nitrogen such as nitrate, urea, ammonium salts, amino acids, microbial growth factors included in one or more of yeast extract, vitamins, and corn steep liquor, a source of assimilable organic or inorganic phosphorous, and a pH buffer such as bicarbonate. Microbial growth factors are currently unspecified compounds which enhance heterotrophic growth of unicellular microorganisms, including fungi and algae. The agar plates can be incubated in the dark at 25-35°C (30°C is preferred) and after 2-4 days numerous colonies will have appeared on the filter. Recovery of 1-5 colonies/plate of the desired organism is not uncommon. Yeast colonies are distinguishable either by color (they frequently are pink) or by their morphology. Yeast colonies are smooth whereas the desired organisms form in colonies with rough or textured surfaces. Individual cells of the desired organism can be seen through a dissecting microscope at the colony borders, whereas yeast cells are not distinguishable, due to their smaller size. Mold and higher fungi colonies are distinguishable from the desired organisms because they are filamentous, whereas the desired organisms are non-filamentous. Clear or white-colored colonies can be picked from the plates and restreaked on a new plate of similar media composition. While most of the desired organisms are clear or white-colored, some are orange or red-colored due to the presence of xanthophyll pigments and are also suitable for selection and restreaking. The new plate can be incubated under similar conditions, preferably at 30°C and single colonies picked after a 2-4 day incubation period. Single colonies can then be picked and placed in, for example, 50ml of liquid medium containing the same organic enrichments (minus agar) as in the agar plates. These cultures can be incubated for 2-4 days at 30°C with aeration, for example, on a rotary shaker table (100-200 rpm.). When the cultures appear

to reach maximal density, 20-40ml of the culture can then be harvested by centrifugation or other suitable method and preserved, as by lyophilization. The sample can then be analyzed by standard, well-known techniques including gas chromatography techniques to identify the fatty acid content of the strain. Those strains with omega-3 highly unsaturated fatty acids can thereby be identified and cultures of these strains maintained for further screening.

Promising strains can be screened for temperature tolerance by inoculating the strains into 250ml shaker flasks containing 50ml of culture media. These cultures are then incubated for 2 days on the shaker table over any desired temperature range from most practically between 27-48°C, one culture at each 3°C interval. Production can be quantified as the total amount of fatty acids produced per ml of culture medium. Total fatty acids can be quantified by gas chromatography as described above. A similar process can also be employed to screen for salinity tolerance. For salinity tolerance a range of salinities yielding conductivities from 5-40 mmho/cm is adequate for most purposes. Screening for the ability to utilize a variety of carbon and nitrogen sources can also be conducted employing the procedure outlined above. The carbon and nitrogen sources were evaluated herein at concentrations of 5g/l. Carbon sources evaluated were: glucose, corn starch, ground corn, potato starch, wheat starch, and molasses. Nitrogen sources evaluated were: nitrate, urea, ammonium, amino acids, protein hydrolysate, corn steep liquor, tryptone, peptone, or casein. Other carbon and nitrogen sources can be used, the choice being open to those of ordinary skill in the art, based on criteria of significance to the user.

It has been unexpectedly found that species/strains from the genus Thrausochytrium can directly ferment ground, unhydrolyzed grain to produce omega-3 HUFAs. This process is advantageous over conventional

fermentation processes because such grains are typically inexpensive sources of carbon and nitrogen. Moreover, practice of this process has no detrimental effects on the beneficial characteristics of the algae, such as levels of omega-3 HUFAs.

The present process using direct fermentation of grains is useful for any type of grain, including without limitation, corn, sorghum, rice, wheat, oats, rye and millet. There are no limitations on the grind size of the grain. However, it is preferable to use at least coarsely ground grain and more preferably, grain ground to a flour-like consistency. This process further includes alternative use of unhydrolyzed corn syrup or agricultural/fermentation by-products such as stillage, a waste product in corn to alcohol fermentations, as an inexpensive carbon/nitrogen source.

In another preferred process, it has been found that omega-3 HUFAs can be produced by Thraustochytrium or Schizochytrium by fermentation of above-described grains and waste products which have been hydrolyzed. Such grains and waste products can be hydrolyzed by any method known in the art, such as acid hydrolysis or enzymatic hydrolysis. A further embodiment is a mixed hydrolysis treatment. In this procedure, the ground grain is first partially hydrolyzed under mild acid conditions according to any mild acid treatment method known in the art. Subsequently, the partially hydrolyzed ground grain is further hydrolyzed by an enzymatic process according to any enzymatic process known in the art. In this preferred process, enzymes such as amylase, amyloglucosidase, alpha or beta glucosidase, or a mixture of these enzymes are used. The resulting hydrolyzed product is then used as a carbon and nitrogen source in the present invention.

Using the collection and screening process outlined above, strains of unicellular fungi and algae can be isolated which have omega-3 highly unsaturated fatty acid contents up to 32% total cellular ash-free dry

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weight (afdw), and which exhibit growth over a temperature range from 15-48°C and grow in a very low salinity culture medium. Many of the very high omega-3 strains are very slow growers. Strains which have been isolated by the method outlined above, and which exhibit rapid growth, good production and high omega-3 highly unsaturated fatty acid content, have omega-3 unsaturated fatty acid contents up to approximately 10%, afdw.

Growth of the strains by the invention process can be effected at any temperature conducive to satisfactory growth of the strains, for example, between about 15°C and 48°C, and preferably between 25-36°C. The culture medium typically becomes more alkaline during the fermentation if pH is not controlled by acid addition or buffers. The strains will grow over a pH range from 4.0-11.0 with a preferable range of about 5.5-8.5.

When growth is carried out in large vessels and tanks, it is preferable to produce a vegetative inoculum in a nutrient broth culture by inoculating this broth culture with an aliquot from a slant culture or culture preserved at -70°C employing the cryoprotectants dimethylsulfoxide (DMSO) or glycerol. When a young, active vegetative inoculum has then been secured, it can be transferred aseptically to larger production tanks or fermenters. The medium in which the vegetative inoculum is produced can be the same as, or different from, that utilized for the large scale production of cells, so long as a good growth of the strain is obtained.

The inventor found that single-celled strains of the order Thraustochytriales (containing omega-3 fatty acids) isolated and screened by the process outlined above, generally exhibited restricted growth, with vegetative growth terminating after a few generations as predicted by Emerson (1950) and Schneider (1976). However, the inventor found that by maintaining relatively high concentrations of phosphorous (e.g., KH_2PO_4 > 0.2g/l) and/or adding a nutritional supplement (source of fungal growth factors) such as yeast extract

or corn steep liquor (greater than 0.2g/l), continuously growing cultures of these unicellular fungi could be maintained. The ability to maintain growth for more than 2-3 generations in liquid culture is termed herein sustained growth. As a group, strains in the genus Thraustochytrium appear to respond more favorably to phosphate additions than those in the genus Schizochytrium, which appear to need less phosphate. In terms of nutritional supplements supplying fungal growth factors, corn steep liquor can be substituted for the yeast extract, and with some strains, has even a more enhanced effect for allowing the strains to achieve high densities in culture. The corn steep liquor and yeast extract contain one or more growth factors necessary for growth of the cells. While the growth factor(s) is not presently defined, it is a component of yeast extract and corn steep liquor, and either of these well-known nutritional supplements are satisfactory. Carbon conversion efficiencies close to 50% (g cell dry weight produced/100g organic carbon added to culture medium) can easily be achieved employing this process.

A microbial product high in protein and high in omega-3 highly unsaturated fatty acids can be produced by harvesting the cells in the exponential phase of growth. If a product significantly higher in lipids and omega-3 highly unsaturated fatty acids is desired, the culture can be manipulated to become nutrient limited, preferably, nitrogen limited for a suitable time, preferably in the range from 6 to 24 hours. The cultures can be transferred to a nitrogen-free medium or, preferably, the initial nitrogen content of the growth medium can be provided such that nitrogen becomes depleted late in the exponential phase. Nitrogen limitation stimulates total lipid production while maintaining high levels of omega-3 highly unsaturated fatty acids as long as the induction period is kept short, usually 6-24 hours. This phase of the culture, when the culture population has achieved its maximum

cell density, is known as the stationary phase. Length of the induction period can be manipulated by raising or lowering temperature, depending on the strain employed. Additionally, the cells can be cultured on a continuous basis in a medium with a high carbon-to-nitrogen ratio, enabling continuous production of high lipid content (and high omega-3 content) cellular biomass. The unicellular strains of heterotrophic microorganisms isolated by the screening procedure outlined above, tend to have high concentrations of three omega-3 highly unsaturated fatty acids: C20:5w3, C22:5w3 and C22:6w3 and very low concentration of C20:4w6. The ratios of these fatty acids can vary depending on culture conditions and the strains employed. Ratios of C20:5w3 to C22:6w3 can run from about 1:1 to 1:30. Ratios of C22:5w3 to C22:6w3 can run from 1:12 to only trace amounts of C22:5w3. In the strains that lack C22:5w3, the C20:5w3 to C22:6w3 ratios can run from about 1:1 to 1:10. An additional highly unsaturated fatty acid, C22:5w6 is produced by some of the strains, including all of the prior art strains (up to a ratio of 1:4 with the C22:6w3 fatty acid). However, C22:5w6 fatty acid is considered undesirable as a dietary fatty acid because it can retroconvert to the C20:4w6 fatty acid. The screening procedure outlined in this invention, however, facilitates the isolation of some strains that contain no (or less than 1%) omega-6 highly unsaturated fatty acids (C20:4w6 or C22:5w6).

HUFAs in microbial products, such as those produced by the present process, when exposed to oxidizing conditions can be converted to less desirable unsaturated fatty acids or to saturated fatty acids. However, saturation of omega-3 HUFAs can be reduced or prevented by the introduction of synthetic antioxidants or naturally-occurring antioxidants, such as beta-carotene, vitamin E and vitamin C, into the microbial products.

Synthetic antioxidants, such as BHT, BHA, TBHQ or ethoxyquin, or natural antioxidants such as tocopherols, can be incorporated into the food or feed products by adding them to the products during processing of the cells after harvest. The amount of antioxidants incorporated in this manner depends, for example, on subsequent use requirements, such as product formulation, packaging methods, and desired shelf life.

Concentrations of naturally-occurring antioxidants can be manipulated by harvesting a fermentation in stationary phase rather than during exponential growth, by stressing a fermentation with low temperature, and/or by maintaining a high dissolved oxygen concentration in the medium. Additionally, concentrations of naturally occurring antioxidants can be controlled by varying culture conditions such as temperature, salinity, and nutrient concentrations. Additionally, biosynthetic precursors to vitamin E, such as L-tyrosine or L-phenylalanine, can be incorporated into fermentation medium for uptake and subsequent conversion to vitamin E. Alternatively, compounds which act synergistically with antioxidants to prevent oxidation (e.g., ascorbic acid, citric acid, phosphoric acid) can be added to the fermentation for uptake by the cells prior to harvest. Additionally, concentrations of trace metals, particularly those that exist in two or more valency states, and that possess suitable oxidation-reduction potential (e.g., copper, iron, manganese, cobalt, nickel) should be maintained at the minimum needed for optimum growth to minimize their potential for causing autooxidation of the HUFAs in the processed cells.

Other products that can be extracted from the harvested cellular biomass include: protein, carbohydrate, sterols, carotenoids, xanthophylls, and enzymes (e.g., proteases). Strains producing high levels of omega-6 fatty acids have also been isolated. Such strains are useful for producing omega-6 fatty acids which, in turn, are useful starting materials for

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chemical synthesis of prostaglandins and other eicosanoids. Strains producing more than 25% of total fatty acids as omega-6 fatty acids have been isolated by the method described herein.

The harvested biomass can be dried (e.g., spray drying, tunnel drying, vacuum drying, or a similar process) and used as a feed or food supplement for any animal whose meat or products are consumed by humans. Similarly, extracted omega-3 HUFAs can be used as a feed or food supplement. Alternatively, the harvested and washed biomass can be used directly (without drying) as a feed supplement. To extend its shelf life, the wet biomass can be acidified (approximate pH = 3.5-4.5) and/or pasteurized or flash heated to inactivate enzymes and then canned, bottled or packaged under a vacuum or non-oxidizing atmosphere (e.g., N_2 or CO_2). The term "animal" means any organism belonging to the kingdom Animalia. The term "animal" means any organism belonging to the kingdom Animalia and includes, without limitation, any animal from which poultry meat, seafood, beef, pork or lamb is derived. Seafood is derived from, without limitation, fish, shrimp and shellfish. The term "products" includes any product other than meat derived from such animals, including, without limitation, eggs or other products. When fed to such animals, omega-3 HUFAs in the harvested biomass or extracted omega-3 HUFAs are incorporated into the flesh, eggs or other products of such animals to increase the omega-3 HUFA content thereof.

It should be noted that different animals have varying requirements to achieve a desired omega-3 HUFA content. For example, ruminants require some encapsulation technique for omega-3 HUFAs to protect these unsaturated fatty acids from breakdown or saturation by the rumen microflora prior to digestion and absorption of the omega-3 HUFAs by the animal. The omega-3 HUFA's can be "protected" by coating the oils or cells with a protein (e.g., zeain) or other substances

which cannot be digested (or are poorly digested) in the rumen. This allows the fatty acids to pass undamaged through the ruminant's first stomach. The protein or other "protectant" substance is dissolved in a solvent prior to coating the cells or oil. The cells can be pelleted prior to coating with the protectant. Animals having high feed conversion ratios (e.g., 4:1 - 6:1) will require higher concentrations of omega-3 HUFAs to achieve an equivalent incorporation of omega-3 HUFAs as animal with low feed conversion ratios (2:1 - 3:1). Feeding techniques can be further optimized with respect to the period of an animal's life that harvested biomass or extracted omega-3 HUFAs must be fed to achieve a desired result.

For most feed applications, the oil content of the harvested cells will be approximately 25-50% afdw, the remaining material being protein and carbohydrate. The protein can contribute significantly to the nutritional value of the cells as several of the strains that have been evaluated have all of the essential amino acids and would be considered a nutritionally balanced protein.

In a preferred process, the freshly harvested and washed cells (harvested by belt filtration, rotary drum filtration, centrifugation, etc.) containing omega-3 HUFAs can be mixed with any dry ground grain in order to lower the water content of the harvested cell paste to below 40% moisture. For example, corn can be used and such mixing will allow the cell paste/corn mixture to be directly extruded, using common extrusion procedures. The extrusion temperatures and pressures can be modified to vary the degree of cell rupture in the extruded product (from all whole cells to 100% broken cells). Extrusion of the cells in this manner does not appear to greatly reduce the omega-3 HUFA content of the cells, as some of the antioxidants in the grain may help protect the fatty acids from oxidation, and the extruded matrix may also help prevent oxygen from readily reaching the fatty acids. Synthetic or natural antioxidants can also

be added to the cell paste/grain mixture prior to extrusion. By directly extruding the cell paste/grain mixture, drying times and costs can be greatly reduced, and it allows manipulation of the bioavailability of the omega-3 HUFAs for feed supplement applications by degree of cell rupture. The desired degree of cell rupture will depend on various factors, including the acceptable level of oxidation (increased cell rupture increases likelihood of oxidation) and the required degree of bioavailability by the animal consuming the extruded material.

The unicellular fungal strains isolated by the method described readily flocculate and settle, and this process can be enhanced by adjusting the pH of the culture to pH $\leq$ 7.0. A 6-fold concentration of the cells within 1-2 minutes can be facilitated by this process. The method can therefore be employed to preconcentrate the cells prior to harvesting, or to concentrate the cells to a very high density prior to nitrogen limitation. Nitrogen limitation (to induce higher lipid production) can therefore be carried out in a much smaller reactor, or the cells from several reactors consolidated into one reactor.

A variety of procedures can be employed in the recovery of the microbial cells from the culture medium. In a preferred recovery process, the cells produced by the subject process are recovered from the culture medium by separation by conventional means, such as by filtration or centrifugation. The cells can then be washed; frozen, lyophilized, or spray dried; and stored under a non-oxidizing atmosphere of a gas such as CO_2 or N_2 (to eliminate the presence of O_2), prior to incorporation into a processed food or feed product.

Cellular lipids containing the omega-3 highly unsaturated fatty acids can also be extracted from the microbial cells by any suitable means, such as by supercritical fluid extraction, or by extraction with solvents such as chloroform, hexane, methylene chloride,

methanol, and the like, and the extract evaporated under reduced pressure to produce a sample of concentrated lipid material. The omega-3 highly unsaturated fatty acids in this preparation may be further concentrated by hydrolyzing the lipids and concentrating the highly unsaturated fraction by employing traditional methods such as urea adduction or fractional distillation (Schlenk, 1954), column chromatography (Kates, 1986), or by supercritical fluid fractionation (Hunter, 1987). The cells can also be broken or lysed and the lipids extracted into vegetable or other edible oil (Borowitzka and Borowitzka, 1988). The extracted oils can be refined by well-known processes routinely employed to refine vegetables oils (e.g. chemical refining or physical refining). These refining processes remove impurities from extracted oils before they are used or sold as edible oils. The refining process consists of a series of processes to degum, bleach, filter, deodorize and polish the extracted oils. After refining, the oils can be used directly as a feed or food additive to produce omega-3 HUFA enriched products. Alternatively, the oil can be further processed and purified as outlined below and then used in the above applications and also in pharmaceutical applications.

In a preferred process, a mixture of high purity omega-3 HUFAs or high purity HUFAs can be easily concentrated from the extracted oils. The harvested cells (fresh or dried) can be ruptured or permeabilized by well-known techniques such as sonication, liquid-shear disruption methods (e.g., French press of Manton-Gaulin homogenizer), bead milling, pressing under high pressure, freeze-thawing, freeze pressing, or enzymatic digestion of the cell wall. The lipids from the ruptured cells are extracted by use of a solvent or mixture of solvents such as hexane, chloroform, ether, or methanol. The solvent is removed (for example by a vacuum rotary evaporator, which allows the solvent to be recovered and reused) and the lipids hydrolyzed by using

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